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# RAPID PCR-BASED MONITORING OF INFECTIOUS ENTEROVIRUSES IN DRINKING WATER

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## ABSTRACT

Currently, the standard method for the detection of enteroviruses and hepatitis A virus in water involves cell culture assay which is expensive and time consuming. Direct RT-PCR offers a rapid and sensitive alternative to virus detection but sensivity is often reduced by PCR inhibitory substances and the requirement for small reaction volumes. Rapid methods for detection of infectious enteroviruses in PCR inhibitory environmental samples are being developed utilising an integrated cell culture/PCR approach (ICC/PCR). With this approach, 300-400l of water were concentrated using charged filters followed by a modified 1l, 1.5% BEV/glycine elution and organic flocculation reconcentration. Water concentrates were analysed by direct RT-PCR, conventional cell culture and ICC/PCR. For ICC/PCR, sample concentrates were incubated with BGM or FRhK cells for 24-48h. The cell culture lysates were collected following freeze-thaw cycles, centrifuged, resin column purified and PCR amplified. In this study viruses known to be present by cell culture analysis could not be detected by direct PCR. Using the integrated method, virus concentrations as low as 0.001MPN/l of original water were detected in samples which were previously inhibitory to direct PCR. In addition, confirmed enterovirus results were achieved as soon as 48h against 5-16d with cell culture alone. Therefore, the integrated approach overcame some of the traditional problems associated with conventional cell culture and direct RT-PCR by allowing rapid, confirmed detection of low levels of enteroviruses in PCR inhibitory samples. © 1997 IAWQ. Published by Elsevier Science Ltd

## KEYWORDS

Enteroviruses; hepatitis A virus; PCR; cell culture; integrated cell culture/PCR; ICC/PCR; inhibition.

## INTRODUCTION

Standard cell culture methods for the detection of human pathogenic viruses in concentrated environmental samples is expensive and time-consuming, requiring up to a month for confirmed positive results (APHA, 1989). Cell culture assay is further complicated by the presence of organic and inorganic materials, in environmental samples, that are toxic to the cell. An alternative method for the detection of viruses in environmental samples is the polymerase chain reaction (PCR), an *in vitro* enzymatic amplification of target nucleic acids which utilises specific oligonucleotide primers (Saiki *et al.*, 1988). Using repeated cycles of PCR, a $10^6$-fold amplification of a single copy of target DNA can be completed within a few hours. The decreased time and cost and increased sensitivity of PCR allow the detection of low numbers of target DNA and RNA usually found in environmental samples; however, one cannot distinguish between amplification of infectious and non-infectious viral sequences. Direct PCR assays have been applied to the detection of

enteroviruses in clinical (Hyypia *et al.*, 1989; Rotbart, 1990) and environmental samples for a more rapid and sensitive test (DeLeon *et al.*, 1990; Pillai *et al.*, 1991; Abbaszadegan *et al.*, 1993). PCR is not without problems, however. For environmental water samples, direct PCR must be able to detect viruses after concentration from large volumes (100-1,000l) of water (APHA, 1989). This is usually accomplished by a filter-adsorption elution method resulting in a concentrate containing viruses, as well as organic and dissolved solids. Concentrated compounds, such as humic substances and metals, can interfere with the activity of the enzymes used in PCR. In addition, the viability or infectivity of viruses in a water sample is an important issue and cannot be determined by direct PCR alone. Although PCR allows the detection of viruses for which no cell line exists for their growth, amplification of non-tissue culture infectious viruses can be misleading since their presence may not be a significant threat to public health. Furthermore, PCR is limited by small reaction volumes (Ma *et al.*, 1995). Typical PCR samples are 10-50µl whereas typical water sample concentrates are 20-30ml. Therefore, it would be impractical to evaluate the entire sample using direct PCR but this can be done easily using cell culture. The use of a combined cell culture/PCR technique utilises the major advantages of each separate methodology while overcoming many of their disadvantages. Samples are first assayed on cells for 24-48h followed by PCR on cell harvests prior to cpe. The combined cultural and molecular techniques increase the PCR equivalent volume which may be examined, reduce the effects of toxicity in cell culture, reduce inhibitory effect on the PCR and, finally, maximise the detection of infectious viruses with the speed and specificity of direct PCR. Previous studies in our laboratory have indicated positive PCR amplification of poliovirus 1 (LSc-2ab) inoculated distilled water after 24h of incubation on BGM cells compared with 3d to more than 2 weeks using cell culture alone (Reynolds *et al.*, 1996). For very low theoretical levels of viruses (<1pfu), cpe was visible only after a second passage on BGM cells. Samples inoculated with 2.8pfu were confirmed positive by PCR after 24h of incubation on BGM cells compared with observation of presumptive cpe after a minimum of 3d. Primary sewage effluent concentrates produced similar results without the inhibition of PCR normally associated with environmental samples. The overall objective of their study was to establish and utilise a new, molecular based protocol to routinely monitor water sources for infectious viruses which may pose a significant public health risk. The hypothesis is that PCR can be used in combination with cell culture techniques to rapidly identify the presence of infectious viral pathogens in environmental waters.

## MATERIALS AND METHODS

***Concentration*** - environmental source waters were filtered through electronegatively charged membrane cartridge filters and eluted with two passes of a 1l volume of 1.5% beef extract V (Becton Dickinson) with 0.05M glycine (pH 9.5) as previously modified (Reynolds *et al.*, 1995). Eluates were then reconcentrated by organic flocculation to a final volume of 20-30ml (Katzenelson *et al.*, 1976). Bacteria were removed from the final samples by centrifugation at 15,000x*g* and treatment with kanamycin, gentamycin sulphate, penicillin G sodium (US Biochemical CO) and nystatin (Sigma) antibiotics, at a final concentration of 100 units/ml. Concentrated samples were stored at -80°C until further analysis.

***Cell culture assay*** - cell monolayers were inoculated with concentrated water samples when stocks were below 100 passages and observed for 10-14d for cpe. Those cells showing cpe were passed once to confirm virus infection. Those cells not showing cpe were also passaged to confirm negativity. Both the first and second passaged material was tested by PCR using appropriate primers. For cell culture assays, BGM and FRhK cells were grown to confluent monolayers in 75cm$^2$ plastic flasks. Before exposure to the sample, the growth medium (5% foetal bovine serum, FBS, for BGM cells and heat inactivating the serum at 56°C for 30min for FRhK cells) was poured off, the monolayers washed twice with Tris-buffered saline solution to prevent toxicity. For each sample, 3ml of concentrate was added to each of five flasks. Samples were incubated at 37°C for 1h while rotating every 15min. 20ml of maintenance medium (2% FBS; 1ml gentamycin 50mg/ml) was added to each flask and the flasks incubated at 37°C being examined daily for 10-14d for cpe. Any flask with suspected viral cpe was confirmed by secondary passage onto fresh monolayers and observed for another 10-14d.

***Direct RT-PCR*** - inhibitory substances were reduced in concentrates prior to PCR using Sephadex G-100 and Chelex-100 spun columns followed by direct RT-PCR (Abbaszadegan *et al.*, 1993; Straub *et al.*, 1994;

Reynolds *et al.*, 1995). PCR enterovirus primers were developed by Abbaszadegan *et al.* (1993) and have been extensively tested in our laboratory in a variety of environmental samples (Straub *et al.*, 1994; Reynolds *et al.*, 1995). The two external 17-20 base primers were designed to amplify a 149-base segment in the single PCR. For confirmatory PCR analysis, a primer sequence internal to the 149bp amplification product was synthesised for use during semi-nested second-cycle PCR. Only sequences amplified from the single PCR reaction are amplified in the semi-nested reaction yielding a 105bp product (Table 1). Primers used for hepatitis A detection were developed by Schwab *et al.* (1991) to amplify a 192bp fragment. Semi-nested primers, developed in our laboratory, have been designed to confirm the single PCR amplification by yielding an internal 100bp product (Table 1).

Table 1. Primers used for PCR

| Virus type | Primer sequence | Product size | Genome position |
|---|---|---|---|
| | 5'-TGTCACCATAAGCAGCC-3' | | 577 - 594 |
| | | 149 | |
| Polio 1 LSc-2ab | 5'-TCCGGCCCCTGAATGCGGCT-3' | | 445 - 465 |
| | | 105 | |
| | 5'-CCCAAAGTAGTCGGTTCCGC-3' | | 530 - 550 |
| | 5'-CAGCACATCAGAAAGGTGAG-3' | | 577 - 594 |
| | | 192 | |
| HAV (HM175) | 5'-CTCCAGAATCATCTCAAC-3' | | 445 - 465 |
| | | 100 | |
| | 5'-GCTTCCCATGTCAGAGTG-3' | | 530 - 550 |

***Integrated cell culture/PCR*** - ICC/PCR was developed in which cell monolayers were inoculated with a water sample concentrate, as described above, and observed for 24-48h. Following limited passage of samples in cell culture, monolayers potentially infected with viruses were lysed, prior to cpe, by freezing and thawing 3x to free any viruses from the infected cells. Lysed cellular product was purified by low speed centrifugation (3,000x*g*, 30min) and Sephadex/Chelex column purification, if needed, prior to PCR analysis. Specific enterovirus or hepatitis A primed PCR was performed on the cell culture lysate followed by confirmatory semi-nested PCR. Initially, PCR was performed on cell harvests, after variable days of incubation, for up to two weeks to standardise incubation times and maximise detection efficiency while minimising experimental costs.

Table 2. Detection of viable enteroviruses in PCR inhibitory water concentrates using ICC/PCR

| | Direct PCR | | | ICC/PCR | | |
|---|---|---|---|---|---|---|
| Sample - (detection sensivity)[1] | Equivalent volume examined L[2] | Theoretical MPN/PCR | Direct PCR[3] | Equivalent volume examined L | MPN/cell culture flask | Integrated PCR results |
| 1 (1) | 0.003 | 2.9 | 3+ | 0.78 | 870 | 3+ |
| 2 (1) | 0.120 | 0.025 | 0 | 36.0 | 7.5 | 3+ |
| 3 (1) | 0.116 | 0.001 | 0 | 34.8 | 0.3 | 2+ |
| 4 (0.1) | 0.082 | 0 | 0 | 24.6 | 0 | 0 |

[1]detection-sensitivity = minimum concentration of control poliovirus inoculum needed for positive RT-PCR result; [2]equivalent volume of original sample examined by either direct RT-PCR or ICC/PCR; [3]amplification products are ranked on a scale of 0-3+ according to band intensity with 3 being the greatest intensity and 0 implying no amplification

## RESULTS

Table 2 dramatically reveals the advantage of the ICC/PCR approach in environmental samples which were highly inhibitory to direct RT-PCR. Samples listed were evaluated by conventional cell culture, direct RT-OCR and the ICC/PCR approach.

Samples positive by conventional cell culture but negative by direct RT-PCR were purified by Sephadex/Chelex column chromatography and low speed centrifugation (above). Concentrates were tested for the level of PCR inhibitory effects by addition of poliovirus 1 at 0.1, 1 and 10pfu. Detection sensitivity of 0.1pfu was the minimum RT-PCR detection limit, when no PCR inhibitory factors were present. If no amplification was observed after adding 0.1pfu, viruses were added at increasing 10-fold increments until a positive result was observed. The increased level of viruses which initiated visible PCR products was noted as the minimum detection sensitivity for that particular sample. Any value greater than 0.1pfu indicated the presence of PCR inhibitory substances. Sample 1 had a high level of viruses originally present (2.9pfu/10μl used in the PCR) and thus was unaffected by the inhibitory factors present. Samples 2 and 3, however, were significantly inhibitory to direct RT-PCR relative to the concentration of viruses present. In addition, equivalent volumes which were examined in the PCR were sufficiently low to create a random occurrence of virus presence in the reaction volume. Virus concentration data in each PCR was theoretically extrapolated from cell culture results on the same sample where larger volumes were evaluated. Notable is that the ICC/PCR approach was consistently correlated with cell cultures with respect to infectious enterovirus presence. Sample 4 was the negative control site in which no enteroviruses were detected by any of the methods used. The integrated approach enabled rapid detection of viruses in PCR inhibitory samples. Although cell culture assays were used, assay times were greatly reduced compared with conventional analysis.

Table 3. Comparison of ICC/PCR and conventional cell culture for detection of HAV

| | Estimated virus concentration (pfu/flask) | Confirmed presence of virus on day | | | | | |
|---|---|---|---|---|---|---|---|
| | | 0 | 3 | 5 | 8 | 11 | 14 |
| Conventional cell culture[1] | 0 | - | - | - | - | - | |
| ICC/PCR | | - | - | - | - | - | - |
| Conventional cell culture | 0.04 | - | - | - | - | - | - |
| ICC/PCR | | - | - | + | + | + | + |
| Conventional cell culture | 0.4 | - | - | - | - | - | - |
| ICC/PCR | | - | - | + | + | + | + |
| Conventional cell culture | 4.0 | - | - | - | - | - | - |
| ICC/PCR | | - | + | - | + | + | + |
| Conventional cell culture | 40 | - | - | - | + | + | + |
| ICC/PCR | | - | + | + | + | + | + |
| Conventional cell culture | 400 | - | - | - | + | + | + |
| ICC/PCR | | - | + | + | + | + | + |

[1]MPN

An added advantage of the ICC/PCR approach was the ability to detect viruses which were infectious but did not produce cytopathic effects or did so very slowly e.g. HAV. Studies of various concentrations of HAV (strain HM175) show a remarkable increase in detection sensitivity of infectious virus using the combined approach and in decreased assay time (Table 3). Virus concentration was determined to be 400, 40, 4, 0.4, 0.04 and 0pfu using FRhK cells (Cromeans *et al.*, 1987). Virus detection limit was defined as 4pfu by plaque assay compared with 40pfu using the MPN method (APHA, 1989). ICC/PCR proved more sensitive than cultural methodologies alone by at least two orders of magnitude with slow growing viruses such as HAV. The detection limit of the combined technique was 0.04pfu. No virus could be detected at this low level until after d3 of the integrated assay indicating that growth of the virus was necessary prior to

detection using the integrated approach. Similar results were also seen at the 0.4pfu level. In some instances, a random negative result may occur on d5 even though d3 and d8 are positive. At very low levels of virus concentration (≤4.0pfu/ml), each individual cell culture flask may not be inoculated with a virus and thus remain negative when analysed by the integrated approach as well as cell culture alone.

## DISCUSSION AND CONCLUSIONS

Evident from this study is that the integrated cell culture/PCR results correlate closely with the cell culture data but are dramatically different from direct PCR on environmental samples owing to increased equivalent volume which may be examined and/or decreased inhibition. Additionally, confirmed integrated results were available in 1-7d compared with 8-16d with cell cultures alone. Furthermore, results indicate that reported sensitivities of conventional cell cultures are underestimating the true level of infectious virus in a given sample. In comparison to conventional cell culture alone, two or three subsequent assays were needed to confirm infectious enterovirus presence in samples that were previously determined negative (Reynolds *et al.*, 1996). Such additional cell culture assays would be extremely costly and time consuming. Therefore, the integrated approach would eliminate the need for additional cell culture assays while providing maximum sensivity. Results from this study may lead to the development of rapid and routine tests for monitoring water concentrates for the presence of infectious enteroviruses and HAV. ICC/PCR results in a significantly more rapid and sensitive test than cell culture alone since it detects replicating viruses prior to the production of cpe. This eliminates the viability issue that is normally associated with PCR analysis, while increasing the equivalent volume examined and overcoming the effects of inhibitory compounds.

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